

## Is *Testudo wernerii* a distinct species?

Pavel ŠIROKÝ<sup>1</sup> & Uwe FRITZ<sup>2</sup>

<sup>1</sup>Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, CZ-61242 Brno, Czech Republic; e-mail: srokyp@vfu.cz

<sup>2</sup>Museum of Zoology (Museum für Tierkunde), Natural History State Collections Dresden, Königsbrücker Landstr. 159, D-01109 Dresden, Germany; e-mail: uwe.fritz@snsd.smwk.sachsen.de

**Abstract:** Sequence variation of a 1066 bp long mtDNA fragment (cytochrome *b* gene, adjacent part of tRNA-Thr gene) of four known-locality samples of *Testudo kleinmanni* (Tripolitania, Libya) and of four samples of *T. wernerii* (Negev, Israel) is compared with additional five sequences of pet trade tortoises allegedly representing *T. kleinmanni*. Four haplotypes, differing in one to four mutation steps occur. The most common haplotype was shared by all known-locality samples of *T. kleinmanni* and three *T. wernerii*. Sequence variation within each nominal species and in the pooled sample of *T. kleinmanni*, *T. wernerii* and pet trade tortoises is the lowest known for any *Testudo* species. We conclude there is no support for the validity of *T. wernerii* Perälä, 2001.

**Key words:** *Testudo kleinmanni*; *Testudo wernerii*; mtDNA; sequence variation; systematics; taxonomy; Israel; Libya

### Introduction

Alpha-taxonomy of western Palaearctic tortoises (*Testudo* spp.) became a much debated field in recent years. Based on differences in shell shape, size and scutellation, many species and subspecies were newly described or resurrected (Chkhikvadze & Tuniyev 1986; Weissinger 1987; Chkhikvadze 1988; Highfield & Martin 1989a, b, c; Chkhikvadze et al. 1990; Highfield 1990; Chkhikvadze & Bakradze 1991, 2002; Mayer 1992; Bour 1996; Perälä 1996, 2001, 2002a, b, c; Peh 2001; Peh & Perälä 2002, 2004; Vetter 2002). Distinctness of most of these taxa was later not confirmed by genetic methods however (van der Kuyl et al. 2002, 2005; Harris et al. 2003; Fritz et al. 2005, 2006, 2007). Many supposedly diagnostic morphological characters correlate with population-specific, and not taxon-specific, differences, suggesting considerable phenotypic plasticity (Harris et al. 2003; Carretero et al. 2005; Fritz et al. 2005, 2006, 2007).

In this paper we compare for the first time mtDNA sequence variation of *Testudo kleinmanni* Lortet, 1883, a species recognized as distinct for more than a century, and the newly described *T. wernerii* Perälä, 2001. *Testudo kleinmanni* and *T. wernerii* are distributed over a patchy, relictual range along the southern Mediterranean coast from Tripolitania, Libya, to the Negev Desert in southern Israel (Perälä 2003a, b). Originally, *T. wernerii* was considered as an isolated population of *T. kleinmanni*, occurring in Egypt east of the Nile Delta and the Negev Desert, Israel. Like in other cases, the species description of *T. wernerii* (Perälä 2001) was based on statistical analyses of morphometric character

ratios, allowing distinction from *T. kleinmanni* in the multivariate space. Recently, *T. wernerii* was placed into the synonymy of *T. kleinmanni* because there are no definite diagnostic characters and natural history features of both nominal species are identical (Baha el Din 2006).

### Material and methods

Using the primers and lab procedures described in Fritz et al. (2007), we sequenced an mtDNA fragment of four Libyan *T. kleinmanni* and four *T. wernerii* comprising most of the cytochrome *b* gene and part of the adjacent tRNA-Thr gene. The *T. kleinmanni* are now housed in the herpetological collection of the National Museum Prague (NMP6V 72917/1–4). These tortoises were collected in the early 1980s by Jiří Zych in Tripolitania and kept alive until their natural death. Exact locality data are available (foothills of Jabal Nefusa, approx. 50 km south of Tripoli; near Mediterranean coast, 50–60 km east of Tripoli) but could not be assigned with certainty to particular individuals. Eli Gefen kindly made samples of *T. wernerii* available. These tortoises are kept in the live collection of the National Museum of Natural History at Tel Aviv University, Israel, and come from the north-western Negev Desert (Fig. 1). In addition to these known-locality specimens, we sequenced one sample from a pet trade tortoise determined as *T. kleinmanni* (voucher: Museum of Zoology, Dresden, MTD 46047) and used four additional sequences of *T. kleinmanni* retrieved from GenBank (accession numbers AJ888370–71, Fritz et al. 2005; DQ080048, Parham et al. 2006; DQ497323, Le et al. 2006) for further analyses. Like our pet trade tortoise, sequences from GenBank are lacking locality data. Sequences were aligned using BIOEDIT 7.0.5.2 (Hall 1999). Considering the very low level of detected sequence variation and that

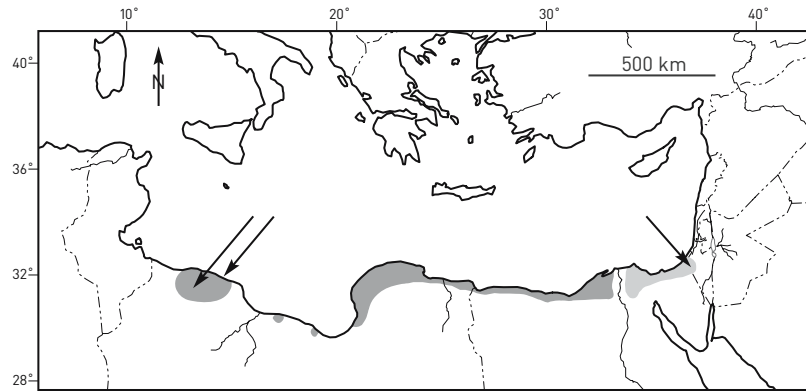


Fig. 1. Ranges of *Testudo kleinmanni* (dark grey) and *T. wernerii* (light grey), based on Schleich et al. (1996), Fritz & Buskirk (1997), Bringsøe & Buskirk (1998), Perälä (2003a, b), Baha el Din (2006) and Schneider & Schneider (2006). Arrows indicate approximate collection sites of known-locality samples.

Table 1. MtDNA sequences of *Testudo kleinmanni* and *T. wernerii* and their corresponding haplotypes.

Sample/Sequence	Geographic Origin	Haplotype	Accession Number	Source
K-1	Tripolitania, Libya	H2	AM98189	This study
K-2	Tripolitania, Libya	H2	AM98190	This study
K-3	Tripolitania, Libya	H2	AM98191	This study
K-4	Tripolitania, Libya	H2	AM98192	This study
W-1	Negev, Israel	H3	AM98193	This study
W-2	Negev, Israel	H2	AM98194	This study
W-3	Negev, Israel	H2	AM98195	This study
W-4	Negev, Israel	H2	AM98196	This study
P-1	Unknown	H4	AJ888370	Fritz et al. (2005)
P-2	Unknown	H4	AJ888371	Fritz et al. (2005)
P-3	Unknown	H2	DQ080048	Parham et al. (2006)
P-4	Unknown	H1	DQ497323	Le et al. (2006)
P-5	Unknown	H2	AM98197	This study

Key: K – *T. kleinmanni*; W – *T. wernerii*; P – pet trade tortoise, allegedly *T. kleinmanni*.

most *T. kleinmanni* and *T. wernerii* shared the same haplotype, we did not repeat previous phylogenetic analyses for *T. kleinmanni* (van der Kuyl et al. 2002; Fritz et al. 2005, 2007; Le et al. 2006; Parham et al. 2006). Instead, we calculated a parsimony network with TCS 1.21 (Clement et al. 2000) and analysed sequence variation using DNASP 4.10.8 (Rozas et al. 2003); uncorrected *p* distances were computed with MEGA 3.1 (Kumar et al. 2004).

## Results

The thirteen sequences of *T. kleinmanni* and *T. wernerii* represent four haplotypes, differing in one to four mutation steps only (Fig. 2, Table 1). The most common haplotype H2 ( $n = 9$ ) was found in all four Tripolitanian *T. kleinmanni* and in three out of the four *T. wernerii*; it is identical with two sequences of pet trade tortoises (AM98197, this study; DQ080048, Parham et al. 2006). The most distinct haplotype (H4,  $n = 2$ ), differing in two to four mutation steps from other haplotypes, corresponds to two earlier published sequences from pet trade tortoises (AJ888370–71, Fritz et al. 2005). Each of the remaining haplotypes H1 and H3 was recorded only once; H1 is the sequence DQ497323 of Le et al. (2006) and H3 was found in one of our samples of *T. wernerii* (AM98193). H1 differs from H3 and the common H2

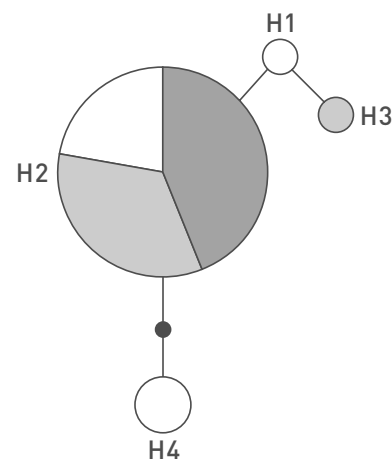


Fig. 2. Parsimony network (TCS 1.21) displaying diversity of mtDNA haplotypes of *Testudo kleinmanni* and *T. wernerii*. The haplotype (H1) with the biggest outgroup probability is on the top. Symbol size corresponds approximately to haplotype frequency; a missing haplotype is indicated by a small black circle. Haplotype frequencies, H1:  $n = 1$ , H2:  $n = 9$ , H3:  $n = 1$ , H4:  $n = 2$ . Dark grey percentages correspond to *T. kleinmanni*; light grey, *T. wernerii*; white, pet trade tortoises. Lines between symbols and missing haplotype indicate one mutation step each.

in one mutation step each. The biggest outgroup probability (0.4878), under coalescent theory (Crandall et

al. 1994) indicative of the oldest haplotype, has H1. Of the four variable sites, one was a singleton; three are parsimony-informative. Three changes were synonymous and one was a replacement change. Haplotype (gene) diversity, Hd was 0.562 (SD = 0.153) and nucleotide diversity per site, Pi was 0.002. The analysis of the pairwise differences distribution (mismatch distribution) resulted in a bimodal curve, indicative for stationary, not expanding populations, as expected in a tortoise with a patchy range of small isolates. This conclusion was also supported by D\* and F\* statistics (Fu & Li 1993). Using an alignment of 1066 bp (1039 bp cytochrome *b* gene; 27 bp tRNA-Thr gene), average uncorrected *p* distance within all sequences was 0.177% (0–0.706%; haplotypes H1–H4), the four known-locality sequences of *T. kleinmanni* were identical (haplotype H2), and average uncorrected *p* distance within *T. wernerii* was 0.176% (0–0.353%; haplotypes H2, H3). Average uncorrected *p* distance between the known-locality samples of *T. kleinmanni* and *T. wernerii* was 0.089% (0–0.354%).

## Discussion

Most of our known-locality samples of *Testudo kleinmanni* and *T. wernerii* share the same mtDNA haplotype (H2). The single unique haplotype of *T. wernerii* (H3) differs only in two mutation steps from the common H2. This is in the same range of variation as observed in the samples of *T. kleinmanni* and the pet trade tortoises (that are likely to represent *T. kleinmanni*). Sequence variation within *T. kleinmanni*, *T. wernerii* and the pooled sample of both nominal species and pet trade tortoises is the lowest known for any *Testudo* species; average sequence divergence within *T. wernerii* (0.176%) corresponds to that observed in the pooled sample (0.177%). Uncorrected *p* distances within other species range between 0.220% (0–1.101%, *T. marginata* Schoepff, 1792, including ‘*T. weissingeri* Bour, 1996’) and 3.346% (0–7.953%, *T. graeca* L., 1758; Fritz et al. 2006, 2007; all cytochrome *b* gene). Likewise is the uncorrected *p* distance between *T. kleinmanni* and *T. wernerii*, 0.089% (0–0.354%), the lowest sequence divergence between any *Testudo* species. When other species are compared, uncorrected *p* distances range between 6.847% (6.452–7.301%, *T. kleinmanni* vs. *T. marginata*) and 12.662% (10.562–15.087%, *T. graeca* vs. *T. hermanni* Gmelin, 1789; Fritz et al., 2007).

Considering this lacking differentiation between *T. kleinmanni* and *T. wernerii* and that mtDNA variation accurately reflects species borders in other *Testudo* species (Fritz et al. 2005, 2006, 2007), we concur with Baha el Din (2006) that *T. wernerii* is a junior synonym of *T. kleinmanni*. The wide distribution of haplotype H2, occurring in the westernmost and easternmost parts of the range of *T. kleinmanni* (Libya and Israel), suggests that the species’ current patchy distribution is the result of recent range interruptions, most likely coinciding with the Holocene aridization of North

Africa and the Near East. The subtle morphological differences of Israeli and other *T. kleinmanni*, thought to be species-diagnostic by Perälä (2001), represent merely another example of population-specific variation and phenotypic plasticity in *Testudo* species.

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